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Hypochlorous acid-induced oxidative damage of human red blood cells: effects of *tert*-butyl hydroperoxide and nitrite on the HOCl reaction with erythrocytes

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Abstract

Hypochlorous acid, one of the most powerful biological oxidants, is believed to be important in the pathogenesis of some diseases. The purpose of this study was to further characterise the membrane and intracellular events which resulted in HOCl-induced oxidative impairments and haemolysis of human erythrocytes and interaction of different oxidative agents, which accumulated during respiratory burst, in the process of RBS oxidation. The sequence of cellular events after red blood cell exposure to HOCl: cell morphological transformations, oxidation of cellular constituents, enzyme modifications, and haemolysis have been evaluated. It was shown that HOCl-treated cells underwent colloid-osmotic haemolysis, preceded by rapid morphological transformations and membrane structural transitions. The activation energy of the process of haemolysis (after removal of the excess of oxidative agent) was estimated to be 146 ± 22 kJ/mol at temperatures above the break point of Arrhenius plot (31–32 °C). This value corresponds to the activation energy of the process of protein denaturation. Modification of erythrocytes by HOCl inhibited membrane acetylcholinesterase (uncompetitive type of inhibition), depleted intracellular glutathione, activated intracellular glutathione peroxidase, but did not induce membrane lipid peroxidation. The presence of other oxidants, nitrite or *tert*-butyl hydroperoxide (*t*-BHP), promoted the oxidative damage induced by HOCl and led to new oxidative reactions.

Keywords: Erythrocyte; Haemolysis; Hypochlorite; Nitrite; Oxidative stress

1. Introduction

The respiratory burst of activated phagocytes (neutrophils and monocytes) both in vitro and in vivo results in the generation of $O_2^{\cdot -}$ and H_2O_2 and the release of the enzyme myeloperoxidase [1]. This enzyme catalyses the reaction of chloride oxidation by H_2O_2 to give the powerful oxidant hypochlorous acid (HOCl). Oxidant concentrations reaching 200 μmol l^{-1} have been reported in some tissues [2]. HOCl plays an important role in bacterial cell killing, but excessive production of HOCl causes tissue damage. This is believed to be important in certain diseases, atherosclerosis and inflammatory conditions [3,4]. HOCl reacts readily with a multitude of biological molecules [5–8].

Proteins are the major targets for HOCl [5]. Treatment of proteins with HOCl results in alterations of amino acid side chains, protein fragmentation and dimerization [5,6]. The majority of protein amino acid residues react with HOCl via free amino groups which results in the formation of semistable chloramine intermediates (RNHCl, mainly lysine-derived chloramines) [6]. Reaction of hypochlorous acid with the double bonds of unsaturated lipids produces α,β-chlorohydrin isomers. Fatty acid acyl and cholesterol chlorohydrins are formed after exposure of red cell membranes to HOCl [7]. The membrane effects of lipid chlorohydrins can contribute to the cytotoxicity of hypochlorous acid [7]. HOCl is able to penetrate the cell membrane and oxidize intracellular thiols. Reduced gluta-

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thione (GSH) is one of the most preferred biological substrates of myeloperoxidase-derived hypochlorous acid [8,9]. Exposure of human red blood cells (RBCs) to low doses of HOCl results in the loss of intracellular GSH [8]. GSH oxidation by HOCl is a highly selective process and precedes oxidation of membrane thiols, formation of chloramines and haemoglobin (Hb) oxidation in RBC [8]. Vissers and Winterbourn [8] have shown that in erythrocytes most of the GSH is converted into glutathione disulphide (GSSG). In addition to GSSG, two stable products of reaction of GSH with HOCl, glutathione sulphonamide and glutathione thiolsulphonate, were identified [9]. Whereas GSSG in the cell is recycled enzymatically, formation of these higher oxidation products is likely to be irreversible [8,9].

While the reactions of hypochlorous acid with cell constituents (proteins, GSH, lipids) are well investigated, the mechanisms of HOCl-mediated cell damage are less understood. The sequence of events after cell exposure to neutrophil generated HOCl, which resulted in cell impairment and death (the molecular mechanism(s) of cytotoxic action of HOCl) is not well known. Schraufstatter et al. [10] have shown the oxidation of different targets on the membrane of P388D1 tumour cells by low HOCl concentrations (10–20 μmol l^{-1}), but the reactions that are essential for cell lysis were not identified. For RBCs, Vissers et al. [11,12] have demonstrated that exposure to hypochlorous acid results in immediate changes of membrane structure affecting cell deformability and membrane permeability. These effects are followed by gradual cell swelling and K^+ leak and finally haemolysis. Interestingly, haemoglobin was not oxidized under these conditions, indicating that other cell constituents, including membrane proteins, were the main targets of hypochlorous acid [13]. However, the question which membrane (or cell) constituents are responsible for HOCl-induced haemolysis remains open.

There is a great interest in the chemistry and biochemistry of oxidative cell injury, induced by activated neutrophils, and in the interactions of damaging species involved in the respiratory burst [14]. RBCs, lacking protein synthesis machinery, have been used extensively as a model system for investigation of the molecular and cellular mechanisms of neutrophil-mediated cell damage [7–12]. One can suggest similar cellular events produced by HOCl for different kind of cells, for example, loss of glutathione (GSH) and total protein thiol groups, as was shown for human endothelial cells [15] or RBCs [8]. Moreover, erythrocytes are exposed to hypochlorous acid in the bloodstream so understanding of the interaction of HOCl with the erythrocyte seems noteworthy. RBCs modified by HOCl can participate in development of the long-term vascular complications in inflammation; interact with neutrophils or other blood cells in the circulation. The purpose of this study was to further characterise the membrane and intracellular events that resulted in HOCl-induced oxidative impairments and haemolysis of human erythrocytes and interaction of different oxidative agents (active forms of chlorine—HOCl, nitrogen—NO_2^-, oxygen—organic hydroperoxide), which accumulated during respiratory burst in the process of RBS oxidation. We have investigated the cascade of the cellular events produced by exposure of RBCs to hypochlorous acid, which can be responsible for cytotoxic action of this agent: morphological transformations, oxidation of cellular constituents, membrane and cytoplasmic enzyme modification, haemolysis. The process of RBC oxidation by HOCl in the presence of such agents as nitrite or organic hydroperoxide, which can be accumulated in RBC during oxidative stress, has also been studied.

2. Materials and methods

2.1. Chemicals

Sodium hypochlorite (NaOCl), sodium nitrite ($NaNO_2$), *tert*-butyl hydroperoxide (*t*-BHP), 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA) and acetylthiocholine iodide (ATCh) were from Sigma-Aldrich, Germany. All other reagents were from POCh (Gliwice, Poland) and were of analytical grade. All solutions were made with water purified in the Milli-Q system.

2.2. Blood samples

Blood from healthy donors was purchased from the Central Blood Bank in Lodz. Blood was taken into 3% sodium citrate. After removing plasma and the leukocyte layer, erythrocytes were washed three times with cold (4 °C) phosphate buffered saline (PBS: 0.15 mol l^{-1} NaCl, 1.9 mmol l^{-1} NaH_2PO_4, 8.1 mmol l^{-1} Na_2HPO_4, pH 7.4). Erythrocytes were used immediately after isolation.

2.3. The susceptibility of erythrocytes to hypochlorous acid-induced damage

Suspensions of RBC in PBS (haematocrit of 10%) were treated with different concentrations of hypochlorous acid at 22 °C for different times (usually 10 min). Then, the cells were washed three times with excess of cold PBS and resuspended in PBS (haematocrit of 10%). HOCl was added as a single bolus of 25 mmol l^{-1} stock solution of NaOCl in PBS to cell suspension and mixed by vortexing. At pH 7.4, this solution contains a mixture of HOCl and OCl^- at approximately 1:1 ratio and is subsequently referred to as HOCl [12]. The concentration of OCl^- was determined spectrophotometrically using an absorbance coefficient of 350 mol^{-1} l cm^{-1} (292 nm) at pH 9.0 [16]. The susceptibility of erythrocytes to HOCl-induced oxidative damage was measured in terms of the

apparent rate constant of cell haemolysis, accumulation of TBA-reactive species (TBARS), oxidation of intracellular oxyhaemoglobin (oxyHb) and GSH and modification of membrane acetylcholinesterase (AChE, EC 3.1.1.7), and cytoplasmic glutathione peroxidase (GSHPx, EC 1.11.1.9).

The process of haemolysis of erythrocytes treated with HOCl was monitored by Hb release or by changes of RBC suspension integral light scattering intensity. In the first case after various time periods, 50 μl of pretreated HOCl RBC suspension (haematocrit of 10%) was added to 1 ml of PBS and centrifuged ($1000 \times g$, 5 min). The Hb content of the supernatants was measured spectrophotometrically by absorbance at 414 nm. For estimation of 100% haemolysis, the same amount of cells was haemolysed by addition of 1 ml of water. In the second case, the dependencies of integral light scattering intensity of erythrocyte suspension were measured as suspension optical density at 700 nm. Immediately before the measurements of light scattering, cell suspension was diluted by PBS to 250 times (optical density at 700 nm did not exceed 0.4).

2.4. The TBARS assay

The amount of TBARS formed was measured using the method of Stocks and Dormandy [17].

2.5. The concentration of metHb

The concentration of metHb in treated erythrocytes was estimated from visible spectra of RBC haemolysates using the algorithm of Winterbourn [18] based on the measurement of optical densities at 560, 577 and 630 nm.

2.6. The concentration of GSH and protein–glutathione mixed disulphides (GSSP)

The intracellular GSH level was determined by the method of Ellman [19], using the extinction coefficient of 13.6 $mmol^{-1}$ l cm^{-1} (412 nm). GSSP were estimated according to the method described by Rossi et al. [20].

2.7. Enzyme activity measurements

AChE activity was determined according to the method of Ellman et al. [21] using ATCh. Erythrocytes were suspended in 0.145 mol l^{-1} NaCl solution containing 5 mmol l^{-1} potassium phosphate, pH 7.9 at 22 °C (0.1% haematocrit). ATCh concentrations were 20–100 μmol l^{-1}, and the concentration of Ellman's reagent in the samples was 50 μmol l^{-1}. The activity of erythrocyte GSHPx was determined as the amount of GSH oxidised by RBC haemolysates, using Ellman's reagent [22]. The GSHPx substrate concentrations were: 1.43 mmol l^{-1} *t*-BHP and 2.5 mmol l^{-1} GSH. After 10 min of incubation at 37 °C, the reaction was stopped by the addition of cold TCA to a final concentration of 4%. Before GSHPx activity measurements, the Hb in haemolysates was converted into CNmetHb by adding the excess of $K_3[Fe(CN_6)]$ and KCN.

All results are the mean ± S.D. of four to six replicates.

3. Results

3.1. Cell haemolysis

RBCs treated with different concentrations of hypochlorous acid underwent morphological transformations and sequential haemolysis, observed by monitoring the cell suspension turbidity and the haemoglobin release (Fig. 1).

The dependencies of integral light scattering intensity on HOCl concentration at various incubation time showed rapid changes of cell size and shape: cell shrinkage (an increase of scattering intensity), followed by cell swelling and haemolysis (a decrease of scattering intensity). The extent of cell impairment depended on the HOCl concentration and incubation time. As the time of cell contact with oxidant increased, the cell transformations have been observed at smaller concentrations of HOCl. Cell morphological transformations preceded haemoglobin release (Fig. 1, curves 3 and 5).

There are some differences in the parameters of the processes of RBC destruction in the presence of HOCl (the process was monitored immediately after oxidant addition) or after oxidant or its soluble product removal by cell

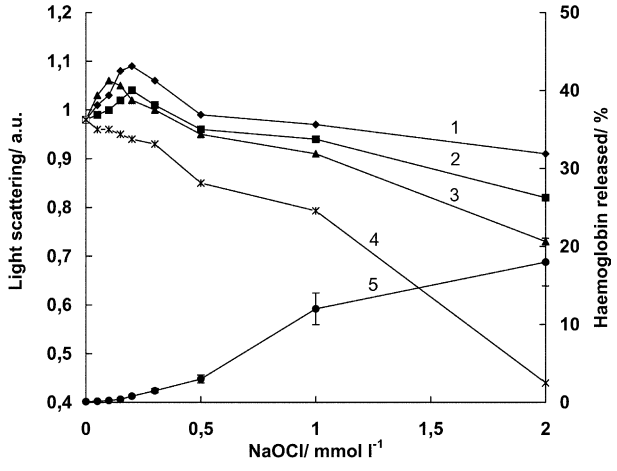

Fig. 1. Lysis of human erythrocytes, induced by HOCl after various time of incubation with oxidant: 2 (1), 15 (2), 30 (3, 5) and 120 min (4) at 22 °C. Dependencies of integral light scattering intensity of erythrocyte suspension (1–4) and of percent of haemoglobin release (5) on oxidant concentration. RBC (10% haematocrit) were incubated with HOCl in PBS, pH 7.4.

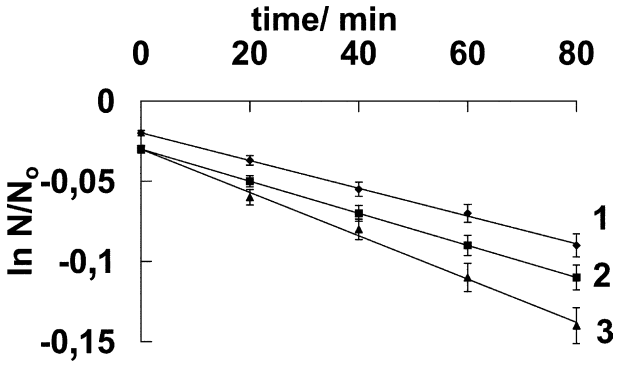

Fig. 2. Time courses of RBC destruction in the presence of 1 mmol l^{-1} HOCl (1) or following oxidant removal after 1 (2) or 10 min (3) of cell incubation with 1 mmol l^{-1} HOCl. PBS, pH 7,4; 22 °C.

washing through different time of exposure to HOCl (post-haemolysis) (Figs. 2 and 3).

The description of the processes of cell destruction may be linearized using the equation $N = N_0 e^{-kt}$, where N_0 and N are the concentrations of the undestroyed cells at the initial moment of time t_o and the moment of time t, respectively, and k is the apparent rate constant of haemolysis. In our experiments, the rate constant of post-haemolysis was higher than that of haemolysis (without cell washing) and, in the case of post-haemolysis, increased as the time of cell treatment with oxidant increased from 1 to 10 min (Fig. 2).

The apparent rate constant of post-haemolysis depended greatly on the haemolytic medium (Table 1). The rate of post-haemolysis increased significantly in potassium-containing medium due to the inhibition of the protective K^+-leakage from damaged cells, and decreased considerably in medium containing sucrose or mannitol, which could not penetrate into the cell.

Fig. 3a shows the dependencies of the rate constant of post-haemolysis on the temperature of cell pretreatment with HOCl (post-haemolysis was measured at a constant temperature of 22 °C). The rate constant significantly decreased and the percent of cells haemolysed during treatment with HOCl increased as the temperature of this exposure increased (Fig. 3a).

From the temperature dependencies of the apparent rate constants of haemolysis, we determined the activation energy of the processes of erythrocyte lysis. The Arrhenius plot showed discontinuity at 31–32 °C (Fig. 3b). The activation energy of haemolysis was found to be 102 ± 15 kJ/mol at the temperatures above the inflection point and 44 ± 5 kJ/mol at the temperatures below the inflection point. The activation energy of post-haemolysis after oxidant removal did not depend on the pretreatment temperature and was 146 ± 22 kJ/mol at the temperatures above the inflection point and 20 ± 4 kJ/mol at lower temperatures (Fig. 3b).

Table 1
The apparent rate constants of post-haemolysis in different haemolytic media

Haemolytic medium	Apparent rate constant, $k \times 10^3/\text{min}^{-1}$
150 mmol l^{-1} NaCl, 10 mmol l^{-1} NaH_2PO_4, pH 7.4	1.28 ± 0.24
150 mmol l^{-1} KCl, 10 mmol l^{-1} NaH_2PO_4, pH 7.4	2.46 ± 0.38
300 mmol l^{-1} sucrose, 10 mmol l^{-1} NaH_2PO_4, pH 7.4	0.15 ± 0.06
300 mmol l^{-1} mannitol, 10 mmol l^{-1} NaH_2PO_4, pH 7.4	0.15 ± 0.06

RBS (10% haematocrit) were treated with 1.0 mmol l^{-1} HOCl in PBS, pH 7.4, at 22 °C for 10 min, washed three times and placed in haemolytic media.

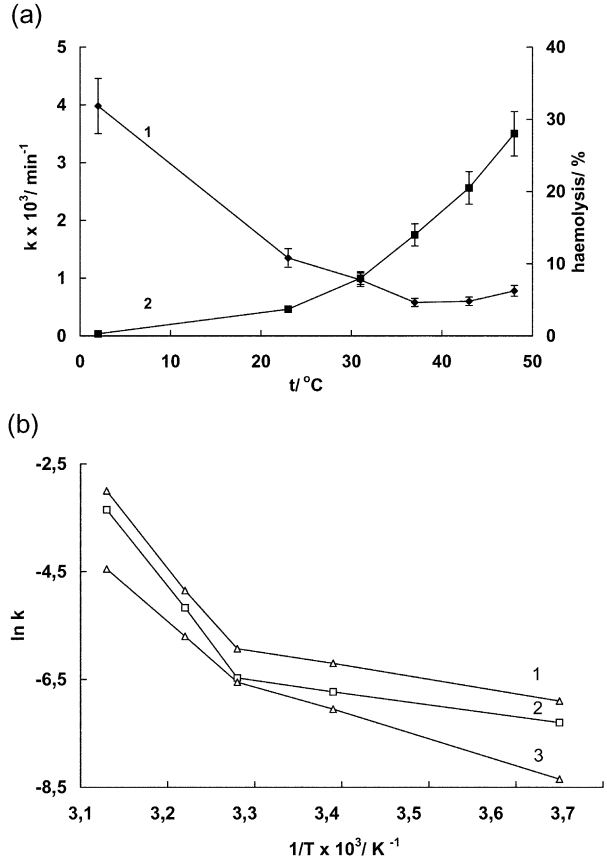

Fig. 3. Effect of temperature on RBC lysis induced by 1 mmol l^{-1} HOCl. (a) The apparent rate constant of post-haemolysis (1) and percent of haemolysis (2) as the functions of temperature of cell treatment with HOCl. The apparent rate constant of post-haemolysis was measured at a constant temperature of 22 °C. (b) Arrhenius plots for the dependencies of the apparent rate constants of post-haemolysis (1, 2) and haemolysis (in the presence of HOCl) (3) on the temperature. Cell suspensions were pretreated with HOCl for 10 min at 2 (1) and 22 °C (2) for measurements of post-haemolysis.

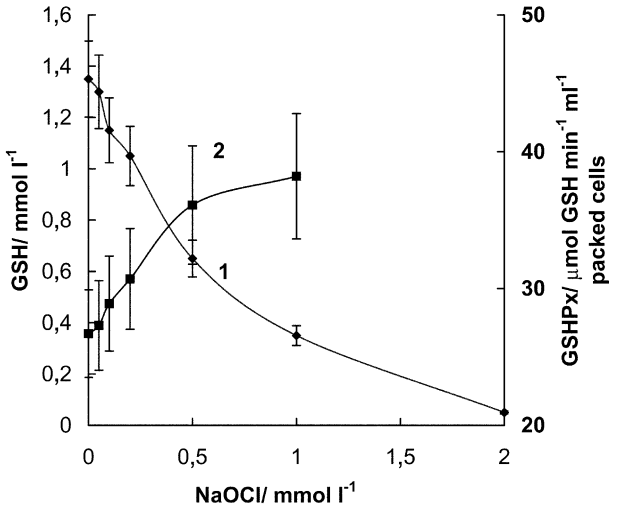

Fig. 4. HOCl-mediated oxidation of intracellular reduced glutathione (1) and modification of GSHPx enzymatic activity (2). RBC suspensions (10% haematocrit in PBS, pH 7.4) were incubated with HOCl for 30 min and then washed prior to measurements of GSH concentration and GSHPx activity.

3.2. Interaction of hypochlorous acid with RBC constituents

RBC incubation with HOCl resulted in oxidation of intracellular reduced glutathione (Fig. 4, curve 1). In the presence of an HOCl scavenger taurine, there was a protection of cellular GSH. Under a ratio of scavenger/oxidant (1:1), we observed almost complete inhibition of intracellular GSH oxidation (Fig. 5).

We have measured the activity of membrane enzyme AChE in erythrocytes exposed to hypochlorous acid as a marker of membrane modification. Lineweaver–Burk plots for AChE activities in intact RBC (curve 1) and in RBC treated with different concentrations of HOCl (curves 2 and 3) are shown in Fig. 6. AChE enzymatic parameters of in- tact RBC were determined: Michaelis–Menten constant $K_m = 0.13 \pm 0.02$ mmol^{-1} l and maximal reaction velocity $V_{max} = 27.9 \pm 3.8$ μmol ATCh min^{-1} ml^{-1} packed cells. HOCl treatment changes the AChE activity in a complex way. For erythrocytes treated with 0.1 mmol l^{-1} HOCl (after removal of oxidant excess), we observed enzyme inhibition which may be described as of partially uncompetitive type (Fig. 6a, curve 2). At higher HOCl concentrations (0.5 mmol l^{-1}), pure uncompetitive type of inhibition was revealed from Lineweaver–Burk plot (Fig. 6a, curve 3). Fig. 6b illustrates enzyme affinity for substrate $K_{m\ app}$ and maximal enzyme velocity $V_{max\ app}$ as a function of HOCl concentration (secondary plot of Lineweaver–Burk plots representing $1/V_{max\ app}$ and $1/K_{m\ app}$). The dependence of $1/V_{max\ app}$ on the concentration of inhibitor was not linear. The RBC treatment with the oxidative agent diminished the $V_{max\ app}$ (a decrease of

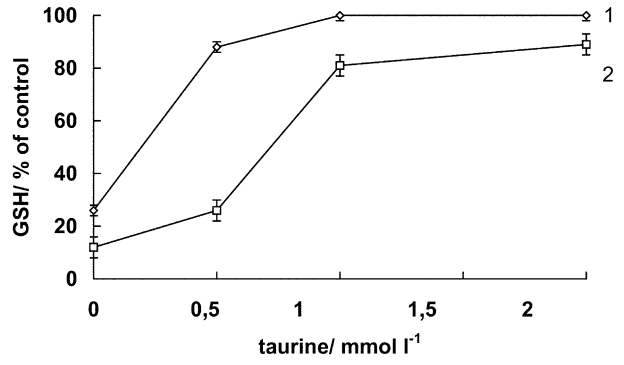

Fig. 5. Inhibition of HOCl-mediated oxidation of intracellular GSH by taurine. Erythrocyte suspension (10% haematocrit in PBS, pH 7.4) was preincubated with taurine for 20 min, then HOCl was added: (1) 0.5 mmol l^{-1} NaOCl; (2) 1 mmol l^{-1} NaOCl. RBC suspensions were incubated with HOCl for 30 min and then washed prior to measurements of GSH concentration.

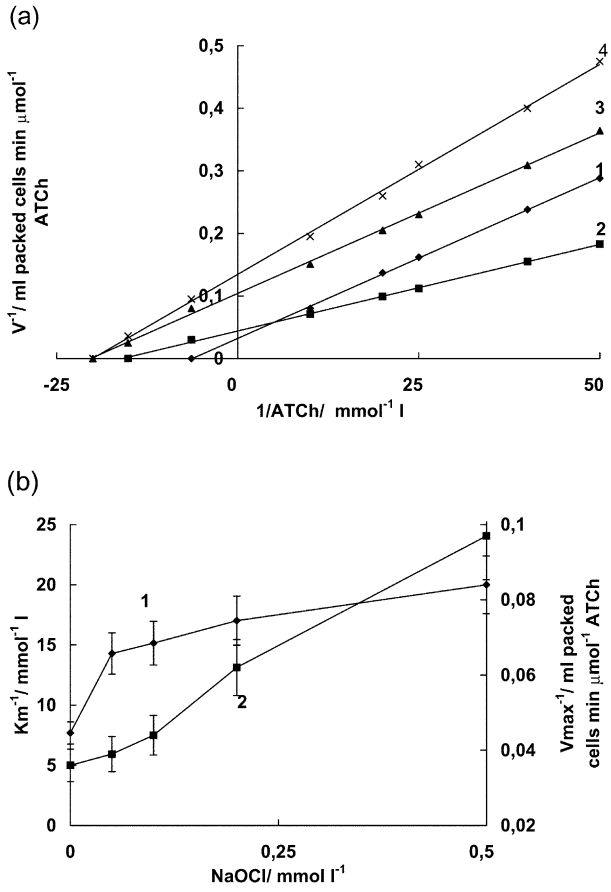

Fig. 6. HOCl-induced inhibition of RBC membrane acetylocholinesterase. (a) Lineaweaver–Burk plot of reciprocal of the initial AChE velocity versus reciprocal of the ATCh concentration: (1) intact cells; (2) cells pretreated with 0.1 mmol l^{-1} HOCl; (3) cells pretreated with 0.5 mmol l^{-1} HOCl; (4) cells pretreated with 1.0 mmol l^{-1} $NaNO_2$ and 0.5 mmol l^{-1} HOCl. (b) Dependencies of reciprocal of the Michaelis–Menten constant K_m (1) and of reciprocal of maximal AChE velocity V_{max} (2) on the HOCl concentration. AChE activity was measured after 30 min of cell incubation with HOCl and subsequent washing.

enzyme catalytic activity) and $K_{m\ app}$ (an increase of substrate affinity), but not in a similar fashion.

Exposure of erythrocytes to HOCl led to activation of intracellular enzyme GSHPx (Fig. 4, curve 2).

Thus, HOCl treatment of RBC led to oxidation of intracellular glutathione and modification of cytoplasmic and membrane enzymes. At the same time, we did not observe any metHb formation even at high (2 mmol l^{-1}) concentrations of HOCl which induced the high extent of cell lysis (Fig. 7a, curve 1). No stable lipid peroxidation endproducts were detectable using TBA assay at any HOCl concentrations (up to 2 mmol l^{-1}) (Fig. 7b, curve 1).

In our experiments, the level of mixed disulphides of GSH with intracellular proteins, mainly Hb, was 82 ± 19 nmol ml^{-1} packed cells and did not increase after cell exposure to HOCl (data not shown).

3.3. The reactions of hypochlorous acid with RBC in the presence of other oxidants: nitrite and hydroperoxide

We also studied the combined action of hypochlorous acid and some other oxidants (nitrite or hydroperoxide) on RBC. As shown before, hypochlorous acid alone did not induce any measured intracellular metHb formation (Fig. 7b). Organic hydroperoxide, *t*-BHP, effectively oxidized intracellular oxyHb to the met form (Fig. 7a). In the presence of hypochlorous acid, the rate of *t*-BHP-induced oxyHb oxidation slightly increased (Fig. 7a, curves 2–3). *t*-BHP alone, in a concentration-dependent manner, induced significant lipid peroxidation in RBC membrane (Fig. 7b). The *t*-BHP/HOCl mixture led to a stronger accumulation of TBARS in cell membranes at 0.5 mmol l^{-1} of *t*-BHP (Fig. 7b, curve 2). At higher hydroperoxide concentrations, there was a slight decrease in the TBARS level in the presence of HOCl (Fig. 7b, curve 3). The apparent rate constant of RBC haemolysis induced by HOCl treatment increased in the presence of different *t*-BHP concentrations (Fig. 7c).

We have studied the effect of another agent, nitrite, on the cell damage, induced by hypochlorous acid. The simultaneous action of nitrite and HOCl slightly increased the percent of haemolysis in comparison to cells treated with HOCl alone (Fig. 8a). As for oxidation of intracellular GSH, we observed additive effect of HOCl and NO_2^- (Fig. 8b). Nitrite is known to be a metHb-forming agent (Fig. 8c, curve 1). Combined treatment of RBCs with NO_2^- and HOCl resulted in a considerable increase of the rate of metHb formation (Fig. 8c, curves 2 and 3). The apparent rate constant of post-haemolysis increased in the case of cell treatment with HOCl in the presence of nitrite (Fig. 8d, curves 2 and 3). No haemolysis was observed after RBC treatment by nitrite (Fig. 8d, curve 1) or *t*-BHP alone (data not shown).

Modification of erythrocytes with NO_2^-/HOCl mixture led to efficient inhibition of AChE by a competitive mechanism in comparison to cell modification with HOCl alone

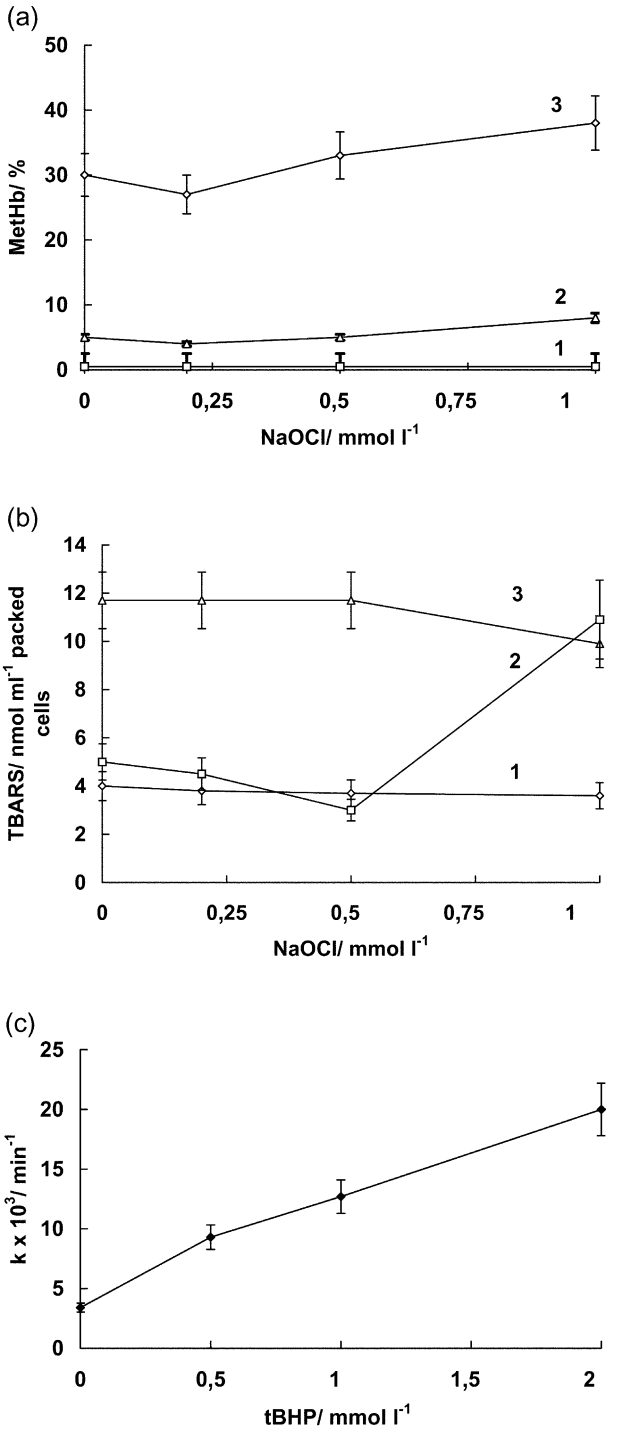

Fig. 7. Effect of *tert*-butyl hydroperoxide on HOCl-induced RBC damage: (a) metHb formation in the absence (1) and in the presence of 0.5 (2) and 1 mmol l^{-1} *t*-BHP (3) (22 °C). (b) Membrane lipid peroxidation (TBARS formation) in the absence (1) and in the presence of 0.5 (2) and 1 mmol l^{-1} *t*-BHP (3) (22 °C). (c) Rate constant of HOCl-induced haemolysis as a function of *t*-BHP concentration (1 mmol l^{-1} HOCl, 28 °C). RBC suspensions (10% haematocrit) in PBS, pH 7.4, were incubated with different concentrations of *t*-BHP (20 min) and then HOCl was added. metHb and TBARS levels were measured after 30 min of cell incubation with HOCl and subsequent washing.

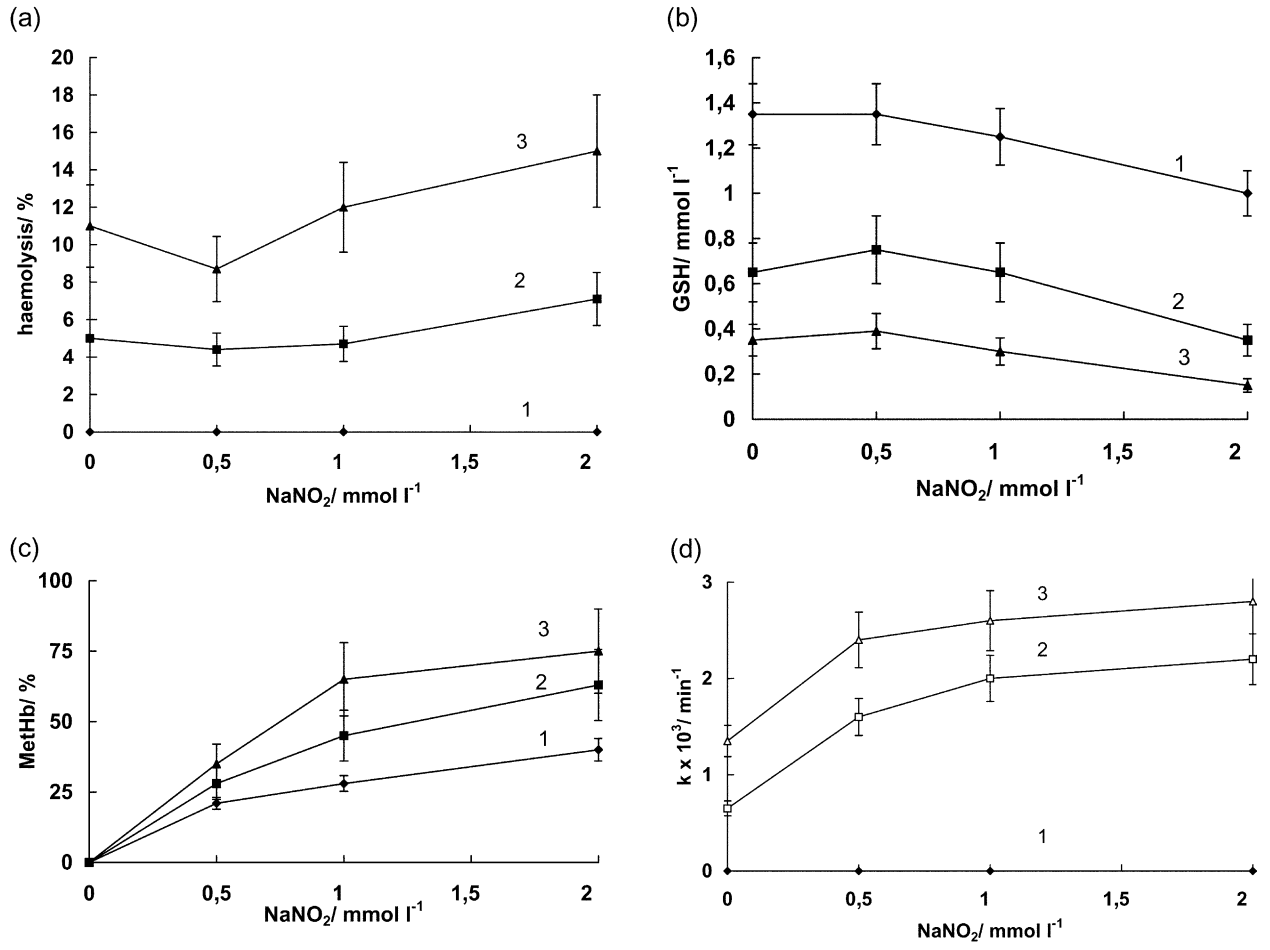

Fig. 8. Effect of nitrite on HOCl-induced oxidative processes: percent haemolysis (a), intracellular GSH level (b), intracellular metHb level (c) and rate constant of post-haemolysis (d) as the functions of $NaNO_2$ concentration in the absence (1) and in the presence of 0.5 (2) and 1 mmol l^{-1} HOCl (3). RBC suspension (10% haematocrit) was incubated with $NaNO_2$/HOCl mixtures in PBS (pH 7.4) for 30 min at 22 °C. Then, the cells were washed and subject to measurements. Nitrite was added immediately before HOCl.

(Fig. 6a, curve 4). Nitrite at these concentrations did not inhibit AChE in our experiments.

4. Discussion

HOCl is an extremely toxic oxidant that can react with a variety of cellular components [5–7]. It has been shown earlier that HOCl caused red blood cell lysis, associated with K^+-leakage and cell swelling, membrane cross-linking, lipid modification [7,8,11,12].

We have studied the membrane and intracellular events in RBC treated with HOCl. Our measurements of light scattering of erythrocyte suspensions, incubated with different concentrations of HOCl, revealed rapid and complex cell morphological transformations: cell shrinkage and subsequent swelling, preceding erythrocyte lysis (Fig. 1).

Our observations, especially the effect of different media on the course of haemolysis, indicate that the HOCl-induced oxidative post-haemolysis is a colloidal-osmotic one. The effects of potassium-, sucrose-, or mannitol-containing media (Table 1) can be rationalised assuming that, like other haemolytic agents, for example, γ-irradiation [23], HOCl induces formation of haemolytic pores in the membrane. The HOCl-induced haemolysis is thought to occur due to disturbance in passive ion permeability, which can be counteracted by active transport processes.

The apparent rate constant of post-haemolysis, which is proportional to the extent of cell damage, depended on the temperature under which cells reacted with HOCl (Fig. 3). In our experiments, the cell stability decreased with the decrease of the temperature of cell treatment with HOCl. The lower stability of cells treated with HOCl at lower temperatures may by due to the lower initial level of chloramines, which retain the oxidising equivalents of HOCl, in the cells treated with HOCl at higher temperatures, or due to the smaller potassium gradient across the cell membrane, or due to a simple decrease of the number of the less stable cells after erythrocyte treatment with HOCl and following cell washing.

One can suggest the possible role of membrane protein modification in erythrocyte membrane destruction. The

formation of protein chloramines as initial products of HOCl reaction with RBC membranes has been proposed earlier [3,6,8,24]. The rate of decomposition of chloramines, once generated on proteins upon treatment with HOCl, increased at higher temperatures [6]. Semistable protein-derived chloramines have been proposed to participate in protein cross-links and fragmentation [6,24] and undertake decarboxylation or deamination yielding aldehyde residues [25]. These processes may cause the erythrocyte membrane damage and pore formation. Chlorination and oxidation of tyrosine and tryptophan residues of proteins yielding chlorotyrosine and indolone derivatives [25] also could be taken into account as possible reactions of HOCl-induced RBC membrane modification. We have previously shown that RBC membrane exposure to hypochlorous acid led to effective oxidation of membrane protein thiol groups, tryptophan residues and chloramines formation, as well as protein peptide bond fragmentation [26]. These events precede cell lysis [26].

The value of activation energy of the post-haemolysis process (146 ± 20 kJ/mol) corresponds to that of protein denaturation [27]. The value of free energy of the pure lipid phase transitions is much lower (20–50 kJ/mol) [28]. The break of the Arrhenius plot at 31–32 °C may reflect RBC membrane transformations due to a phase transition of membrane lipids [29].

Our findings show that the reaction of HOCl with RBC is rapid and leads to irreversible changes in the cell. The damage of a critical membrane protein or proteins by HOCl may be the reason for cell haemolysis. However, the involvement of the lipid phase modification (chlorohydrin formation) and changes of lipid bilayer arrangement in the membrane haemolytic events cannot be omitted. Recently Vissers at al. [12] have showed that non-reducible cross-links with most membrane proteins have been a major result of cell exposure to both HOCl and HOBr and have been closely correlated with haemolysis. At the same time, we found that preliminary denaturation of RBC membrane protein spectrin by heating (48 °C, 20 min) did not change the cell stability (no alterations in the rate of haemolysis, data not shown). Similarly, the anion channel (Band 3 protein) blocker, diisothiocyanostilbene-disulphonate (DIDS), had no effect on HOCl-mediated lysis [8].

The oxidation of intracellular reduced glutathione, as well as membrane thiols, occurred concurrently to cell haemolysis and was not a reason for cell lysis [8,11]. We did not reveal any oxyHb oxidation or additional lipid peroxidation at the HOCl concentrations used, which is consistent with other observations [11]. We observed that hypochlorous acid induced modification of enzymatic activities of erythrocytes. Low doses of HOCl were found to inhibit externally oriented membrane-bound AChE (Fig. 6). It is known that changes of AChE enzyme activity reflect erythrocyte membrane structural transformations (modifications of the membrane lipid fluidity and surface charge) [30]. The AChE activity changes may arise from two types of HOCl effects: direct enzyme modification or disturbances in the complementarity between the protein hydrophobic surface and the lipid environment in the membrane. The mechanism of the inhibition depended on the oxidant concentration used. Probably, membrane (or enzyme) modification by oxidant and membrane structural transitions yielded a non-productive modified enzyme-ATCh complex and prevented the stage of acetylation of the catalytic centre with substrate to form acetylated AChE, as it was shown, for example, in the case of AChE uncompetitive inhibition with malathione [31]. A stronger effect of NO_2^-/HOCl mixture on the membrane was revealed in terms of the membrane AChE inhibition than for nitrite or HOCl alone (Fig. 6a, curve 4). It must be stressed that, in our case, there was a non-reversible type of AChE inhibition by HOCl treatment.

As it is shown in Figs. 7 and 8, the mixtures NO_2^-/HOCl or *t*-BHP/HOCl have stronger effects on the RBC components in comparison to HOCl, *t*-BHP or nitrite alone. Free radicals generated as a result of reactions between the inflammatory mediator HOCl and organic hydroperoxide or nitrite may initiate new oxidative processes in the cell [14,32–34]. Organic hydroperoxides are always present in biological membranes as a result of lipid peroxidation processes. Endothelial cells, as well as phagocytes, generate nitric oxide (NO) which is subsequently oxidized to NO_2^- [32,34]. Hypochlorous acid slightly increased the level of metHb formed in RBC by *t*-BHP treatment and promoted the increase of lipid peroxidation products (at 0.5 mmol l^{-1} *t*-BHP) (Fig. 7a,b). *t*-BHP/HOCl mixture had a considerably more pronounced haemolytic activity (Fig. 6c) as compared with HOCl alone. The effect of oxidant mixture depended on the molar ratio of the oxidants.

It has previously been shown that only HOCl reactions with organic hydroperoxides previously accumulated in lipid systems led to reinitiation of lipid peroxidation [34]. The *tert*-butoxyl radical, formed in the reaction of HOCl and *t*-BHP [33], promoted effective accumulation of TBARS (at a definite molar ratio *t*-BHP/HOCl) (Fig. 7b, curve 2) and participated in membrane impairments and haemolysis (Fig. 7c).

It has been shown that the reaction between neutrophil-derived HOCl and nitrite produces nitrating species such as nitrogen dioxide (NO_2^-), nitryl chloride (NO_2Cl) and nitronium ion (NO_2^+) [14,34]. In our experiments, nitrite only slightly increased the percent of HOCl-induced haemolysis of RBC, but significantly promoted intracellular oxyHb oxidation. Probably, intermediate species of the reaction between NO_2^- and HOCl effectively oxidized intracellular oxyHb and damaged cell membrane (Fig. 8c,d).

In conclusion, HOCl-induced oxidation resulted in rapid RBC morphological transformations and membrane structural transitions preceding haemolysis, intracellular glutathione depletion and cell enzyme modifications. After a

removal of the excess of HOCl, erythrocytes underwent colloidal-osmotic haemolysis, similar to the postirradiation haemolysis (after RBC γ-irradiation) [24]. We assumed that the main reason for HOCl-induced haemolysis is probably membrane protein impairments. In the presence of other oxidants, nitrite or *t*-BHP, HOCl induce some new oxidative reactions resulting in intracellular oxyHb oxidation or membrane peroxidation.

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